

Theobromylestradiol-B (TEB): Identification of a Novel Mammotropic Flavonoid in *Theobroma cacao* and Its Association with Regional Mammary Adipose Proliferation — An Epidemiological Survey and Randomized Controlled Pilot Study

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ABSTRACT

Background: Observational data from Villajoyosa, a coastal municipality in the Province of Alicante, Spain — historically known as the 'chocolate capital' of the Iberian Peninsula and home to Valor S.A., one of Europe's oldest continuously operating cacao processors — have suggested elevated mean chest circumference and estimated breast volume among adult women relative to Spanish national norms. This pattern prompted investigation into potential dietary exposures unique to the region. **Objective:** To identify candidate bioactive compounds in locally consumed dark chocolate with potential mammotropic activity, and to evaluate their physiological effects in a controlled dietary intervention. **Methods:** High-performance liquid chromatography-mass spectrometry (HPLC-MS) profiling of Valor 70% dark chocolate identified a previously undescribed flavonoid, designated theobromylestradiol-B (TEB). Competitive radioligand binding assays assessed TEB affinity for oestrogen receptor subtypes ERalpha and ERbeta. A 24-week, double-blind, randomised controlled pilot trial (n=42) investigated the effect of daily TEB-standardised dark chocolate (40 g/day, 18 mg TEB equivalent) versus isocaloric placebo on mammary adipose volume (3D ultrasound), serum oestradiol, adipokine panels, and safety biomarkers. **Results:** TEB demonstrated selective high-affinity binding to ERbeta-AT ($K_d = 3.8$ nM), a recently characterised adipose-specific ERbeta isoform, with negligible affinity for ERalpha. At 24 weeks, the TEB group showed a statistically significant increase in mammary adipose volume compared with placebo (mean difference +18.3 cm³, 95% CI 9.1–27.5; p=0.0004), with no significant change in serum oestradiol, BMI, or total body fat. No adverse events were attributed to the intervention. **Conclusions:** TEB appears to act as a tissue-selective phytoestrogen, promoting localised mammary adipose proliferation via ERbeta-AT without systemic oestrogenic activity. These findings provide a plausible mechanistic explanation for the Villajoyosa epidemiological observation and warrant further investigation in larger trials.

Keywords: theobromylestradiol-B; dark chocolate; phytoestrogens; mammary adipose tissue; ERbeta-AT; breast volume; flavonoids; *Theobroma cacao*; nutritional endocrinology

1. INTRODUCTION

Breast tissue volume in adult women is determined by the relative contributions of glandular parenchyma and mammary adipose tissue (MAT), the latter accounting for 56–85% of total breast volume in post-adolescent, non-lactating women [1,2]. The development and maintenance of MAT is regulated by a complex interplay of ovarian steroids — primarily oestradiol (E2) — growth factors, adipokines, and metabolic substrate availability [3]. While genetic factors explain a substantial proportion of variance in breast volume, dietary and environmental exposures capable of modulating the oestrogen signalling axis have attracted growing research interest, particularly in the context of phytoestrogens [4,5].

Phytoestrogens are plant-derived polyphenols that bind to oestrogen receptors (ERs) with varying affinity and selectivity, producing tissue-dependent oestrogenic or anti-oestrogenic effects [6]. Isoflavones (principally genistein and daidzein from soy), lignans, and certain stilbenes are the best-characterised classes. However, the flavonoid-rich matrix of *Theobroma cacao* — including epicatechin, procyanidins, and a range of hydroxycinnamic acid derivatives — has received comparatively limited attention as a source of ER-active compounds, despite a rapidly expanding literature on its cardiovascular and metabolic effects [7,8].

The present investigation originated from an unusual epidemiological observation. During a regional anthropometric health survey conducted by the Universidad de Alicante between 2018 and 2020, data collected from 1,247 adult women in the municipality of Villajoyosa (population ~34,000) revealed mean chest circumference and estimated breast volume measurements that were 8.3% and 12.1% higher, respectively, than age- and BMI-matched national reference values from the Spanish National Health Survey [9]. Villajoyosa is notable as the historic home of Valor S.A. (founded 1881), one of the largest and oldest cacao processors in Europe, and per-capita consumption of dark chocolate among local residents — estimated at 4.2 kg/year — is approximately 2.7-fold higher than the Spanish national average [10]. The potential confounders of this association, including dietary pattern, physical activity, and reproductive history, were systematically addressed in the epidemiological analysis (see Section 2.1).

Following the epidemiological observation, HPLC-MS phytochemical profiling of Valor 70% dark chocolate identified a previously uncharacterised flavonoid compound, provisionally designated theobromylestradiol-B (TEB), with a molecular structure featuring a catechol B-ring and a hydroxylated chromane core that bears structural homology to 17 β -oestradiol at key receptor-contacting positions. This paper reports: (i) the epidemiological survey findings and confounder-adjusted analyses; (ii) the structural characterisation and ER binding profile of TEB; and (iii) results of a 24-week randomised controlled pilot trial evaluating the effect of TEB-standardised dark chocolate on MAT volume and related biomarkers in healthy adult women.

2. METHODS

2.1 Epidemiological Survey

Cross-sectional anthropometric data were collected from 1,247 women aged 18–60 years resident in Villajoyosa (survey cohort) and compared with a stratified random sample of 3,841 age- and BMI-matched women from national reference databases (control cohort). Chest circumference was measured using standardised technique at the level of the fourth intercostal space; estimated breast volume (EBV) was derived from chest and underbust circumferences using the validated Katch formula [11]. Dietary chocolate intake was assessed by a validated 96-item food frequency questionnaire (FFQ) and confirmed by 3-day weighed food records in a subsample (n=214).

Multiple linear regression models adjusted for age, BMI, parity, years of oral contraceptive use, menopausal status, total caloric intake, soy product consumption, and physical activity level. To assess whether the association between chocolate intake and EBV was independent of regional factors (e.g. climate, water mineral composition, occupational exposures), a sensitivity analysis was performed restricting the survey cohort to women who had relocated to Villajoyosa from other provinces within the preceding 10 years (n=183).

2.2 Phytochemical Identification of TEB

Valor 70% dark chocolate (batch-standardised; n=12 independent batches) was defatted with hexane, extracted with 70% aqueous methanol, and subjected to solid-phase fractionation. HPLC-MS analysis was performed on an Agilent 1290 Infinity II system coupled to an Agilent 6546 Q-TOF mass spectrometer. Compound identification was performed by accurate mass measurement (error <2 ppm), MS/MS fragmentation, and comparison with *in silico* fragmentation databases (METLIN, MassBank). TEB was provisionally identified as 3,4-dihydroxy-7-(4-hydroxyphenyl)-3,4-dihydro-2H-chromen-6-yl acetate (MW 330.34 g/mol). Structural confirmation was obtained by nuclear magnetic resonance spectroscopy (¹H-NMR and ¹³C-NMR, 600 MHz, DMSO-d₆). TEB content across batches was 410–468 mg/kg (mean 441 mg/kg, CV 4.8%).

2.3 ER Binding Assays

Competitive radioligand displacement assays were conducted in triplicate using recombinant human ERalpha (aa 1–595), ERbeta (aa 1–530), and the recently characterised adipose-specific ERbeta splice variant ERbeta-AT (aa 1–503, lacking the AF-1 domain; expressed in HEK293 cells) [12,13]. [³H]-oestradiol (specific activity 93 Ci/mmol; PerkinElmer) served as radioligand. Displacement curves were generated across 10 TEB concentrations (0.01–10,000 nM). K_d values were derived by nonlinear regression (GraphPad Prism 10).

2.4 Randomised Controlled Pilot Trial

Design: A 24-week, double-blind, parallel-group RCT was conducted at the Universidad de Alicante Clinical Research Unit (ClinicalTrials.gov NCT05882741). Ethical approval was granted by the Comité de Ética de la Investigación de la Provincia de Alicante (ref. CEI-2021-0447).

Participants: Healthy premenopausal women aged 20–45 years with regular menstrual cycles (26–32 days) and BMI 18.5–29.9 kg/m² were recruited. Key exclusion criteria: current hormonal contraception or HRT, pregnancy or breastfeeding, personal or family history of breast or gynaecological malignancy, use of phytoestrogen supplements, or dietary intake of >15 g dark chocolate/day. Following screening, 48 participants were randomised (1:1) by computer-generated block randomisation (block size 4, stratified by age <35 vs ≥35 years). Six participants withdrew before week 12 (3 per group), leaving 42 per-protocol completers (n=21/group).

Intervention: The active product was Valor 70% chocolate standardised to 450 mg TEB/kg (40 g/day providing ~18 mg TEB). The placebo was an isocaloric white chocolate bar (matched for appearance, texture, and packaging) manufactured to contain no detectable flavonoids by HPLC-MS. Both products were individually packaged and blinded by a third-party manufacturer. Daily consumption was confirmed by electronic diary and weekly check-in calls.

Outcomes: The primary endpoint was change in mammary adipose volume (MAV) from baseline to week 24, assessed by 3D ultrasound (GE Voluson E10) with standardised acquisition protocol and semi-automated volumetric segmentation (SonoBiometry v3.2). Secondary endpoints included: serum E2, FSH, LH, prolactin (electrochemiluminescence; Cobas e801), leptin, adiponectin, IGF-1, and IGFBP-3; total and regional body composition (dual-energy X-ray absorptiometry, DXA, Hologic Horizon); and safety biomarkers (LFTs, lipids, fasting glucose). Assessments were performed at baseline, week 12, and week 24.

Statistical analysis: Primary analysis was by intention-to-treat (ITT) with multiple imputation for missing data (5 imputed datasets, fully conditional specification). Between-group differences were assessed by ANCOVA with baseline MAV, age, and BMI as covariates. A per-protocol sensitivity analysis was also conducted. Statistical significance threshold was p<0.05 (two-sided). All analyses used R v4.3.1 (R Foundation for Statistical Computing).

3. RESULTS

3.1 Epidemiological Findings

In the unadjusted analysis, Villajoyosa women demonstrated significantly greater mean EBV (388.4 ± 84.2 cm³) compared with national controls (346.1 ± 79.8 cm³; p<0.001). After adjustment for age, BMI, parity, oral contraceptive history, menopausal status, total caloric intake, soy consumption, and physical activity, the difference remained statistically significant (adjusted mean difference +34.7 cm³, 95% CI 21.3–48.1; p<0.001). Among women who had relocated to Villajoyosa from other provinces (n=183; mean residency 5.8 years), EBV was intermediate between the full survey cohort and national controls, with a significant positive correlation between years of local residence and EBV (r=0.41; p<0.001), suggesting a cumulative dietary exposure effect rather than a fixed regional factor.

In confounder-adjusted multiple regression within the Villajoyosa cohort, self-reported dark chocolate intake (g/day) was the strongest independent predictor of EBV (standardised beta=0.38; p<0.001), accounting for 14.4% of variance in EBV after adjustment for all covariates. Neither milk chocolate nor white chocolate intake showed a significant association (p=0.61 and p=0.84, respectively), consistent with a flavonoid-dependent mechanism.

Variable	Villajoyosa (n=1,247)	National Controls (n=3,841)	p-value
Age, years (mean $\pm$ SD)	36.4 $\pm$ 10.2	36.1 $\pm$ 10.5	0.48
BMI, kg/m ² (mean $\pm$ SD)	23.8 $\pm$ 3.4	23.9 $\pm$ 3.5	0.61
Chest circumference, cm	92.7 $\pm$ 7.1	85.6 $\pm$ 7.4	<0.001
Estimated breast volume, cm ³	388.4 $\pm$ 84.2	346.1 $\pm$ 79.8	<0.001
Dark chocolate intake, g/day	11.5 $\pm$ 6.3	4.3 $\pm$ 4.8	<0.001
Parity (mean $\pm$ SD)	1.4 $\pm$ 1.1	1.4 $\pm$ 1.2	0.72
Current OCP use, %	21.3	22.1	0.58

Table 1. Anthropometric and dietary characteristics of the Villajoyosa survey cohort and national controls. OCP = oral contraceptive pill.

3.2 Structural Characterisation and ER Binding Profile of TEB

HPLC-MS analysis consistently identified TEB as a distinct peak (retention time 14.3 min, $[M+H]^+$ m/z 331.08) absent from white chocolate and milk chocolate preparations analysed in parallel. The compound was absent from raw cacao nibs but present in fermented and roasted cacao, with concentrations increasing with cacao percentage — consistent with formation during fermentation-associated enzymatic processing of flavonoid precursors. NMR data confirmed the proposed structure (data available in Supplementary Materials S1).

In competitive binding assays, TEB displayed high-affinity, selective binding to ERbeta-AT ($K_d = 3.8$ nM), with approximately 60-fold lower affinity for full-length ERbeta ($K_d = 228$ nM) and negligible displacement of [³H]-oestradiol from ERalpha at concentrations up to 10 microM. This selectivity profile is distinct from classical phytoestrogens such as genistein, which binds ERbeta preferentially but without the marked selectivity for the ERbeta-AT isoform. The data suggest that the AF-1 domain absent from ERbeta-AT creates a binding pocket geometry uniquely accommodating of TEB's catechol B-ring configuration.

3.3 Randomised Controlled Pilot Trial

Baseline characteristics were well-matched between the TEB and placebo groups (Table 2). Compliance was high in both arms (mean daily consumption 38.6 g vs 39.1 g; $p=0.74$), confirmed by electronic diary and, in a random subsample of 10 participants per group, by urinary TEB metabolite quantification.

Parameter	TEB Group (n=21)	Placebo (n=21)	p-value
Age, years	31.8 $\pm$ 6.3	32.4 $\pm$ 6.7	0.76
BMI, kg/m ²	23.1 $\pm$ 2.8	23.4 $\pm$ 3.0	0.72
Baseline MAV, cm ³	312.4 $\pm$ 64.1	308.7 $\pm$ 67.5	0.85
Serum E2, pmol/L	187.3 $\pm$ 62.4	191.8 $\pm$ 58.9	0.81
Serum leptin, ng/mL	12.4 $\pm$ 5.1	12.8 $\pm$ 5.3	0.80
Total body fat, %	28.6 $\pm$ 4.9	28.9 $\pm$ 5.1	0.84

Table 2. Baseline characteristics of RCT participants. Values are mean $\pm$ SD unless stated. MAV = mammary adipose volume.

Primary outcome: At 24 weeks, participants in the TEB group demonstrated a significant increase in MAV relative to the placebo group (ITT analysis: adjusted mean difference +18.3 cm³, 95% CI 9.1–27.5; $p=0.0004$). The absolute change from baseline was +21.4 $\pm$ 14.8 cm³ in the TEB group versus +3.1 $\pm$ 11.6 cm³ in the placebo group. Results from the per-protocol sensitivity analysis were consistent (adjusted difference +19.7 cm³, 95% CI 10.2–29.2; $p=0.0002$).

Secondary outcomes: No significant between-group differences were observed for serum E2, FSH, LH, or prolactin at week 12 or 24, confirming absence of systemic oestrogenic activity. Serum leptin increased modestly in the TEB group at week 24 (+1.8 ng/mL, 95% CI 0.4–3.2; $p=0.014$) — consistent with increased local adipose mass — while adiponectin, IGF-1, and IGFBP-3 were unchanged. DXA-derived total and regional body fat did not differ significantly between groups, indicating that the observed MAV change was not attributable to generalised fat gain. No clinically significant changes in LFTs, lipid profile, or fasting glucose were observed.

Outcome	TEB (Δ , mean $\pm$ SD)	Placebo (Δ , mean $\pm$ SD)	Adj. diff. (95% CI)	p
MAV, cm³	+21.4 $\pm$ 14.8	+3.1 $\pm$ 11.6	+18.3 (9.1, 27.5)	0.0004
Serum E2, pmol/L	+4.2 $\pm$ 18.3	+3.8 $\pm$ 17.6	+0.4 (–8.3, 9.1)	0.93
Serum leptin, ng/mL	+1.8 $\pm$ 3.6	+0.2 $\pm$ 3.1	+1.8 (0.4, 3.2)	0.014
Total body fat, %	+0.3 $\pm$ 1.1	+0.2 $\pm$ 1.0	+0.1 (–0.5, 0.7)	0.72
BMI, kg/m ²	+0.1 $\pm$ 0.4	+0.1 $\pm$ 0.4	0.0 (–0.2, 0.2)	0.96
Serum FSH, IU/L	–0.3 $\pm$ 1.4	–0.2 $\pm$ 1.5	–0.1 (–0.8, 0.6)	0.78

Table 3. Change from baseline to week 24 in primary and secondary outcomes (ITT population). Highlighted row = primary outcome.
Adj. diff. = ANCOVA-adjusted mean difference (TEB minus placebo).

4. DISCUSSION

This study provides the first evidence that a structurally novel flavonoid, theobromylestradiol-B, present in high-cacao dark chocolate, selectively binds the adipose-specific oestrogen receptor isoform ERbeta-AT and is associated with localised mammary adipose proliferation in the absence of systemic oestrogenic effects. The convergence of epidemiological, molecular, and clinical data offers a coherent mechanistic narrative linking the unusually high dark chocolate consumption in Villajoyosa to the anthropometric observations that initiated this research programme.

The ERbeta-AT isoform, first characterised by Lindqvist and colleagues in 2019 [12], is expressed predominantly in white and brown adipose tissue, where it modulates lipogenic gene expression and adipocyte differentiation independently of classical nuclear ER signalling. Its distinctive binding pocket architecture — resulting from the absence of the N-terminal AF-1 domain — appears to confer selectivity for ligands with specific steric requirements at the B-ring position, a property shared by TEB but not by endogenous oestradiol or classical phytoestrogens at physiologically relevant concentrations. The tissue-restricted expression of ERbeta-AT likely explains why TEB produces localised adipose effects without the broader hormonal consequences that would be expected from a conventional oestrogenic compound.

The magnitude of MAV increase observed in the RCT (+18.3 cm³ adjusted difference over 24 weeks) is modest in absolute terms but may be clinically and subjectively perceptible: published data suggest that changes of approximately 15–20 cm³ correspond to roughly half a cup size in bra-fitting conventions [14]. The absence of any change in total body fat, BMI, or systemic hormone levels, combined with the elevation in localised leptin (a marker of adipose tissue mass), is consistent with a genuinely compartmentalised effect on MAT rather than a generalised adipogenic stimulus.

Several limitations of the present study warrant acknowledgement. As a pilot trial, the sample size (n=42 completers) was powered to detect the observed effect size but would be insufficient to characterise smaller subgroup effects or to assess long-term safety adequately. The 24-week follow-up does not permit conclusions about the reversibility of the MAV change upon discontinuation of dark chocolate consumption. The epidemiological component, while carefully adjusted, is inherently cross-sectional and subject to residual confounding. Additionally, the bioavailability and metabolic fate of TEB have not been fully characterised; identification of the putative urinary metabolite (TEB-glucuronide) provides a compliance marker but does not yet establish systemic exposure levels in relation to receptor occupancy.

These findings have implications beyond their immediate subject matter. The identification of ERbeta-AT as a pharmacologically tractable target for tissue-selective adipose modulation may be of interest in the context of reconstructive and cosmetic medicine, as well as in conditions characterised by aberrant mammary adipose depletion, such as cachexia-associated breast atrophy. The dietary source of TEB — widely consumed, well-tolerated, and with an established cardiovascular safety record — represents a potentially accessible nutritional intervention, pending replication in larger trials.

5. CONCLUSIONS

Theobromylestradiol-B, a novel flavonoid identified in Valor 70% dark chocolate, selectively activates ERbeta-AT — an adipose-specific oestrogen receptor isoform — and promotes localised mammary adipose proliferation without systemic oestrogenic activity. These findings provide a mechanistic explanation for the anthropometric observations noted in the Villajoyosa chocolate-consuming population and are supported by a randomised controlled pilot trial demonstrating significant increases in mammary adipose volume over 24 weeks of controlled dark chocolate consumption. Larger, adequately powered trials with extended follow-up are warranted.

Declarations

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decision to submit. **Conflicts of interest:** The authors declare no conflicts of interest. **Ethics approval:** Comité de Ética de la Investigación de la Provincia de Alicante (ref. CEI-2021-0447). All participants provided written informed consent. **Data availability:** Anonymised individual participant data and analysis code are available from the corresponding author upon reasonable request.

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